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Assessment of the genetic structure of European bison (*Bison bonasus* L.) from Bialowieza by single nucleotide substitutions of DRB3 and DQB genes of major histocompatibility complex

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Abstract: Differences in SNP-substitutions of the major histocompatibility complex (MHC) *DRB3* and *DQB* genes involved in the immune response were shown in the genetic structure of the Belarusian and Polish populations of European bison. Unique and rare alleles, which are valuable for the population, and will contribute to an increase in genetic diversity and the viability of the species, were identified.

Key words: European bison, *Bison bonasus*, frequency of *DRB3* and *DQB* genes, major histocompatibility complex

Introduction

European bison (*Bison bonasus L.*) is a single wild species of the Bovinae subfamily in Europe that survived up to now. At present E. bison has a status of "a restored species", vulnerable (VU) in the IUCN Red List, included in Annex III of the Bern Convention, Red Data Books of Russia, Poland, Ukraine, and Lithuania. European bison seems now to be saved from extinction. At the same time survival of the species in historical prospect remains under the threat.

In the past, E. bison occupied vast areas of broad-leaved and mixed forests in Europe (except for the countries located in its northern part), Caucasia, Transcaucasia and North Iran. *Bison bonasus* was subdivided into 3 subspecies: *Bison bonasus bonasus* – Lowland (Bialowieza) bison, (currently the Lowland line is derived from 7 founders); *Bison bonasus caucasicus* – Caucasian bison, the Lowland-Caucasian line originates from 12 founders, including only one representing this subspecies and remaining animals belonging to the Lowland subspecies; *Bison bonasus hungarorum* – Transylvanian-Carpathian mountain bison, extinct. Of E. bison subspecies surviving to the present times, only Lowland bison (Bialowieza) was preserved in its pure form. The last representative of Transylvanian-Carpathian mountain bison

disappeared in the XVII-th century. The Caucasian subspecies was exterminated in the XX-th century, and is now represented by crossbreds with *Bison b. bonasus* subspecies.

The "bottleneck" stage endured by E. bison population has resulted in an increase of the inbreeding level and spread of recessive alleles in its populations. Inbreeding could reduce the overall viability of animals and even lead to the extinction of the species as a whole (O'Brien and Evermann 1988; Kozlo and Bunevich 2009).

For the recovery of the genetically depleted population, a male "Kavkaz" from the Caucasian subspecies was brought to Bialowieza Forest in the early XIX-th century (Kozlo and Bunevich 2009; Sipko 2002). This bull has produced 7 calves (3 males and 4 females) with Bialowieza females during its life. These animals and their progeny were caught and brought to Bialowieza Forest.

At present the largest E. bison population of the world, inhabiting the Bialowieza Forest is divided into two parts – Belarusian and Polish. Those subpopulations have however a common origin. The results obtained by us through the microsatellite analysis show that despite a common origin, and high similarity of Belarusian and Polish European bison populations, probably their isolation have led to obvious differences in the genetic structure. The occurrence of unique alleles of microsatellite *loci* confirms the difference of the Belarusian population (Mikhailova and Medvedeva 2013).

The genetic potential of present E. bison is greatly depleted, with degeneration symptoms in the Bialowieza line and increased susceptibility of animals to infectious diseases being observed in Belarus (Kozlo and Bunevich 2009). Reduction in genetic diversity of genes responsible for formation of the immune response is known to increase the population sensitivity to pathogens. Infectious diseases are considered one of the primary causes of rare species extinction among wild animals. Inbreeding depression, associated with the "bottleneck" stage endured by the population, can reduce the ability of individuals to induce immune response due to the loss of variability of genes responsible for the resistance to infections. It refers to highly polymorphic genes of the vertebrate major histocompatibility complex (MHC), initiating an immune response. Therefore, one of the strategic trends of breeding Bialowieza bison is implementation of the activities aimed at increase of this population heterogeneity owing to reduction in the probability of loss of one or more MHC gene alleles.

The major histocompatibility complex is a group of genes, and their encoded protein receptors located on the cell surface. The MHC gene complex of the *Bovidae* family, to which E. bison *(Bison bonasus)* and domestic cattle (*Bos taurus*) belong, is located on a short arm of the 23rd autosomal chromosome, consists of 3500 bp and contains over 220 genes (Abbas *et al.* 2007). They play a crucial role in recognition of alien agents and immunity development. Antigen-representing molecules, encoded

by the MHC, belong to classical genes of classes I, II and III. The major histocompatibility complex molecules of the class II are encoded by a separate set of genes. These genes are located near the centromere and include several loci (*DPA*, *DPB*, *DQA*, *DQB*, *DRA*, *DRB*).

The main features of the MHC complex are its significant polygeny, i.e. the presence of several nonallelic genes, the protein products of which have a similar structure and perform identical functions, as well as pronounced polymorphism – the presence of many allelic forms of the same gene. Preservation of the MHC gene diversity is a key element of the program efficiency for conservation and breeding of rare and endangered animal species (Hughes 1991).

Each of the MHC gene alleles provides an opportunity to respond to a certain set of antigen peptides. Therefore, individuals having heterozygous genotypes of the MHC genes are able to induce more effectively an immune response to the effect of multiple antigens, so they are more likely to resist infections.

The MHC gene sites, encoding extracellular domains and forming a groove (peptide-binding site) where antigens of intracellular or extracellular pathogens are bound, exhibit the highest polymorphism. This polymorphism is mainly concentrated in the second exon of the MHC genes of class II.

Wegner *et al.* (2003) have revealed a positive relationship between a variety of pathogens inhabiting an organism and the diversity of the MHC gene alleles in a population. The authors have assumed that the level of infectious load has an impact on polymorphism of the MHC genes at the population level. A group of scientists from Canada has advanced a theory that polymorphism of the MHC gene of class II – the degree of diversity depends on the latitude of the population habitat. In northern latitudes, the gene diversity in populations is higher than in southern ones (Mainguy 2009).

Sharp reduction of population numbers endured by the population, the so called "bottleneck" stage, may lead to restriction of the MHC gene diversity, and increase the species vulnerability as described in the cheetah (Mainguy 2009). However, some species continue to exist despite low polymorphism and even monomorphism caused by the "bottleneck" effect in the past (O'Brien *et al.* 1985; O'Brien and Evermann 1988; Mikko 1995). The authors come to the opinion that the MHC system is only one of many protective systems against pathogenic infections, and its polymorphism level does not exert a high effect on long-term survival of a population. These findings verify the role of balancing breeding as a mechanism for maintaining the MHC gene variability in natural populations.

This article presents studies on polymorphism of the MHC *DRB3* and *DQB* genes. For conducting further breeding work on the conservation of E. bison diversity, it is necessary to consider the genetic potential of all populations – as a possible source of new allelic variants of genes. In our study, the genetic structures of European bison of Belarusian and Polish populations were compared.

Materials and Methods

We have analyzed 56 biological samples of individuals from the Belarusian population and 30 of the Polish population of genes of the major histocompatibility complex DQB and DRB3 genes. The standard method of salt extraction with some modifications (Mikhailova and Medvedeva 2013) was used for DNA extraction (Zinovieva *et al.* 2002). Direct sequencing of the PCR gene fragments is the most common method for describing their nucleotide sequence and revealing polymorphism. However, due to the presence of multiple alleles at the *locus*, sequencing may not be informative – nucleotide sequences of different allelic variants are superimposed on each other. To solve this problem it is necessary to clone the amplified fragments into the vector, with only one allele being ligated into the plasmid. We have used the pGEM-3Zf (+) and pBluescript IIKS/SK (+) plasmids as vectors. Amplification for increasing the T-ends was carried out with the amplifier of the "Bio-Rad" Company (Germany) at 72°C for 2 h. The restricted sites were purified by Gel Extraction Kit (Fermentas, Lithuania).

Characteristics of the PCR primers, used for amplifying fragments of both MHC genes, are given in Table 1. The amplified sites cover the region of the 1st intron – 2nd exon of the investigated genes.

Table 1. Characteristic of the primers used for amplifying fragments of the *DRB3* and *DQB* genes in the major histocompatibility complex

Locus	Primer	Primer sequence (5'-3')	Amplicon size (bp)	Reference
DRB3	HLO30-F	ATCCTCTCTCTGCAGCACATTTCC	284	Abbas *et al.* 2007
	HLO32-R	TCGCCGCTGCACAGTGAAACTCTC		
DQB	DQB-F	TCCCCCGCAGAGGATTTCGTG	217	Donald and Traul 2005
	DQB-R	CGCACTCACCTCGCCGCTGC		

Polymerase chain reaction was carried out in 10 μL of the mixture, containing 5–10 ng of genomic DNA, 10 pM of each primer, 50 mM $MgCl_2$, 4 mM dNTP, 1× PCR-buffer, 1,7 U of Taq-polymerase (Primetech, Belarus). Amplification was carried out with the automatic programmed thermal cycler of the "Bio-Rad" Company (Germany) under the following temperature conditions: 95°C – 15 min.; 35 cycles – 94°C – 30 sec., 55°C – 30 sec., 72°C – 30 sec.; we have extended final elongation up to 15 min. at 72°C for increasing A-ends for ligation into the vector. Each *locus* was amplified independently in a separate tube.

Reaction products were visualized on 1.5% agarose gel using molecular weight marker Gene Rulertm 1 kb DNA Ladder. Prior to ligation PCR-products were purified by DNA Gel Extraction Kit (Fermentas, Lithuania). Then cloning of the gene fragment into the vector was conducted. For this purpose the PCR-product of 217

/ 284 bp was ligated into the obtained T-vector. The ligase mixture contained: 20 ng of the T-vector, 20 ng of the insertion, 1 μL 10× ligase buffer, 5U T4-ligase and water was added to the final volume of 10 μL H_2O.

Then the ligase mixture was used for transformation of *E. coli bacterium*, strain XLBlue (Maniatis 1984).

For selecting colonies, carrying the vector with the insertion, the blue-white screening method was used. For that, IPTG and X-Gal were added to the liquid LB-medium. The vector carries a part of the *lacZ* (*lacZα*) gene, the other part of this gene is found in bacterial chromosome. The product of this gene causes the blue color of the colonies, carrying the vector, on the medium containing X-Gal and IPTG.

However, when ligating the insertion into the vector, the *lacZα* sequence is interrupted, as far as it contains a multiple cloning site. Thus, the cells carrying the vector with the insertion, form white unstained colonies. The colonies, selected in such way, were subcultured into individual Petri dishes with solid LB-medium containing ampicillin.

Screening of the obtained colonies for the presence of the insertion was conducted by PCR with the standard M13 primers to the plasmid multiple cloning site (Fig.1).

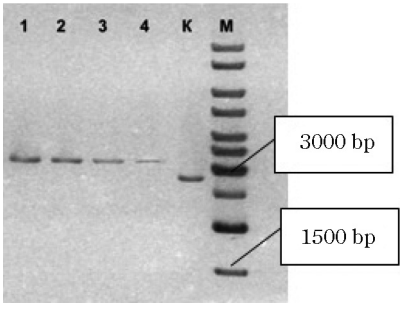

Fig. 1. Electrophoregram of colony screening for the presence of the insertion with the M13 primers: 1–4 – Samples: vector (2 886 bp) + insertion (393 bp), K – control, vector without insertion (2 886 bp), M – marker

As shown in Fig.1, the insertion of the gene fragment is visualized on the electrophoregram with 3279 bp band (№ 1–4).

Sequencing of the obtained clones was performed on both chains of the fragment using the Big Dye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, USA) with 3500 Genetic Analyzer (Applied Biosystems, USA).

Results and Discussion

We have analysed polymorphism of the 2nd exon of the major histocompatibility complex *DRB3* gene in European bison individuals of the Belarusian population from the National Park "Bialowieza Forest".

Table 2. Comparison between allele frequancies of the major histocompatibility complex *DRB3* gene in Belarusian and Polish populations of E. bison.

	Number of individuals	**Bibo DRB3*-0101**	**Bibo DRB3*-0201**	**Bibo DRB3*-0301**	**Bibo DRB3*-0401**
Belarusian population	56	0,563	0,384	0,053	0
Polish population (acc. Radwan *et al.* 2007)	172	0,346	0,270	0,364	0,020

Out of four alleles of the MHC *DRB3*gene described for European bison (Radwan 2010; Radwan e*t al.* 2007; Tokarska 2011; Medvedeva and Mikhailova 2011) three alleles – Bibo-DRB3*-0101, Bibo-DRB3*-0201, Bibo-DRB3*-0301 were found in the Belarusian population. The occurrence frequencies of each allele vary significantly (Table 2). Thus, allele Bibo-DRB3*-0401 was not found in the Belarussian population, while in Polish population it occurs with a frequency of 0.02. Frequency of allele Bibo-DRB3*-0301 in the Bielorusian population was 0,053. The frequency of this allele was 0,364 in Polish population.

It was revealed that the most rare Bibo-DRB3*-0301 allele in the Belarusian population is widespread in the Polish one. The Bibo-DRB3*-0401 allele is rare in the Polish population, its occurrence frequency is 2%. It was however not identified in the sample analysed by us (Medvedeva and Mikhailova 2011).

Such a number of allelic variants of the *DRB3* gene is low compared to the diversity of allelic variants described for cattle (*Bos taurus*, 105 alleles), American bison (*Bison bison*, 15 alleles) and other ungulate animals such as red deer (*Cervus elaphus*), goats and sheep. This fact is a consequence of a sharp reduction in the number of E. bison population in the early twentieth century. Other ungulate species, which were subject to the "bottleneck" effect, also show a limited diversity of the MHC genes, e.g. moose (*Alces alces*) (Mikko *et al.* 1995).

Polymorphism of the major histocompatibility complex *DQB* gene was first studied by us in European bison. The nucleotide sequence of the 2nd exon of the MHC *DQB* gene was investigated in 30 individuals from Belarusian and 30 individuals from Polish populations of European bison. The obtained *DQB* gene fragment is 217 bp.

Three allelic variants of the *DQB* gene: Bibo-DQB-Bel1, Bibo-DQB-Bel2 and Bibo-DQB-Bel+Pol1 were identified in the Belarusian population (Fig.2).

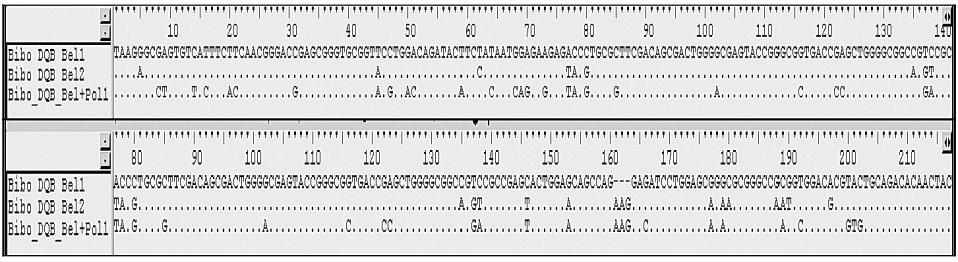

Fig. 2. Nucleotide sequences of allelic variants of the MHC *DQB* gene in the Belarusian E. bison population

Allelic variants differ from each other in a number of nonsynonymous substitutions (Fig. 5). The Bibo-DQB-Bel1 allele is also characterized by deletion of three nucleotides (161–163 bp) that entails loss of 54 codon – lysin amino acid (Fig. 3).

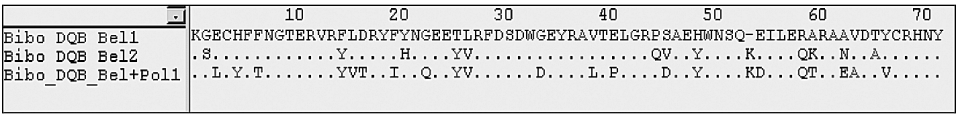

Fig. 3. Comparison of amino acid sequences determined by allelic variants of the MHC *DQB* gene in the Belarusian E. bison population

Three allelic variants of the *DQB* gene – Bibo-DQB-Bel+Pol1, Bibo-DQB-Pol2, Bibo-DQB-Pol3 were identified in the Polish population (Fig. 4).

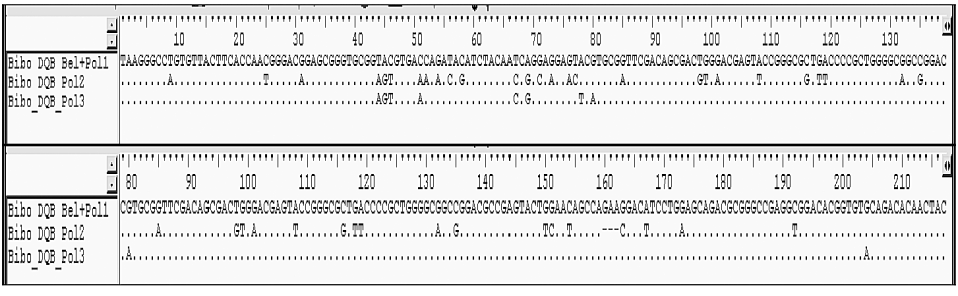

Fig. 4. Nucleotide sequences of allelic variants of the MHC *DQB* gene in the Polish E. bison population

Allelic variants identified in the Polish bison population have also differences in nucleotide sequences. The Bibo-DQB-Pol2 allele, just as the Bibo-DQB-Bel1 allele in the Belarusian population, has a deletion of three nucleotides in position 160 bp that leads to the loss of 53 codon – glutamine amino acid (Fig. 5).

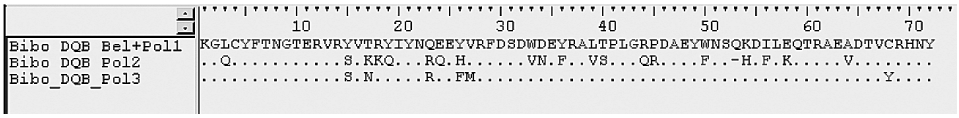

Fig. 5. A comparison of amino acid sequences of the peptide-binding site of the MHC *DQB* gene in the Polish European bison population

It is known that many of the ungulates have a common group of alleles, in which there is such a deletion (Radwan et *al.* 2007).

Distribution of the occurrence frequencies in allelic variants of the *DQB* gene in Belarusian and Polish populations is presented in Table 3.

Table 3. Comparison between allele frequancies of the major histocompatibility complex *DQB* gene in Belarusian and Polish European bison populations

	Number of individuals	**Bibo-DQB-Bel1**	**Bibo-DQB-Bel2**	**Bibo-DQB-Bel+Pol**	**Bibo-DQB-Pol2**	**Bibo-DQB-Pol3**
Belarusian population	30	0,672	0,083	0,245	–	–
Polish population	30	–	–	0,850	0,083	0,067

Five allelic variants of this gene were detected, of them 2 are typical for only the Belarusian population (Bibo-DQB-Bel1, Bibo-DQB-Bel2), 2 – for only the Polish one (Bibo-DQB-Pol2, Bibo-DQB-Pol3), and there is 1 allelic variant (Bibo-DQB-Bel+Pol) in both populations.

In the Belarusian E. bison population, the highest frequency was revealed for the *DQB* Bibo-DQB-Bel1 allele – 67.2%. The second allelic variant, typical for only the Belarusian individuals, Bibo-DQB-Bel2, has a frequency of 8.3%. The allele of the *DQB* Bibo-DQB-Bel+Pol1 gene, present in both populations, has a frequency of 24.5% in the Belarusian population, and 85% in the Polish one. Bibo-DQB-Pol2 and Bibo-DQB-Pol3 alleles occur in Polish population with the frequency of 8.3% and 6.7% respectively.

Three groups of clusters – cluster I, II; III, IV; and V are clearly distinguished in the phylogenetic tree in the graphical representation of the allelic variants of the DQB gene in individuals from the Belarusian and Polish populations (Fig. 6).

Clusters I and II were formed by the Bibo-DQB-Bel1 and Bibo-DQB-Bel2 allelic variants. The DQB gene alleles, identified in the individuals belonging to the Polish population, form clusters III and IV – Bibo-DQB-Pol3, Bibo-DQB-Pol2, respectively. The Bibo-DQB-Bel+Pol1 allelic variant, detected in the representatives of both populations, forms one cluster V.

A high difference in the available allelic gene variants (many nonsynonymous substitutions) is considered to be maintained during natural selection. The individuals with higher allelic diversity of the MHC genes have a selective advantage, since they can form an immune response to a wider range of antigenes.

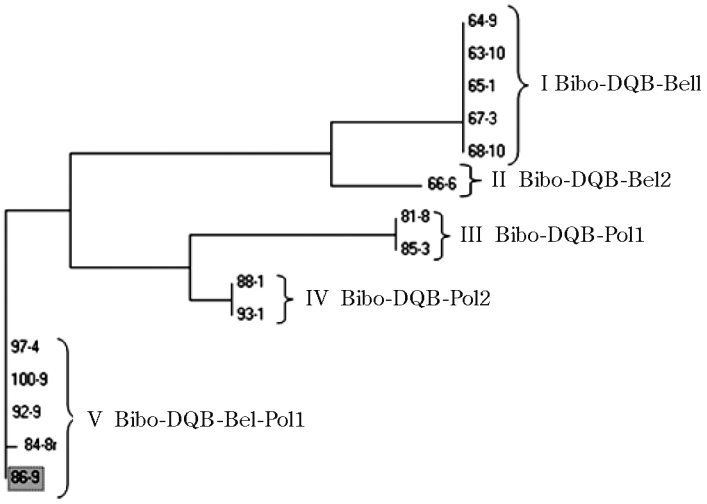

Fig. 6. Genetic differences in the allelic variants of the MHC DQB gene in individuals from Belarusian and Polish European bison populations

Thus, the differences in the frequencies of the allelic variants of the major histocompatibility complex DRB3 and DQB genes were reflected in the genetic structure of the Belarusian and Polish European bison populations. The Bibo-DRB3*-0301 allele of the DRB3 gene with a very low frequency was detected in the Belarusian European bison population. The unique allelic variants of the Bibo-DQB-Pol2 and Bibo-DQB-Pol3 genes, which are valuable for increasing genetic diversity of the Belarusian population, were identified also in the Polish population.

Detection of individuals carrying unique allelic variants of microsatellite loci and the MHC genes will contribute to an increase in genetic diversity, and involvement of unique genes and alleles in the breeding process, that will certainly increase the viability of a species.

The study on populations of various animal species with molecular-genetic methods is an important stage in the development of measures for biodiversity conservation. Use of phylogenetic analysis allows us to develop more effective measures for the protection of rare and endangered species as well as to apply optimal schemes of economic use of natural resource i.e. wild fauna species.

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Ocena struktury genetycznej żubrów z Puszczy Białowieskiej przy pomocy pojedynczych substytucji w genach DRB3 i DQB głównego kompleksu zgodności tkankowej

Streszczenie: W pracy przedstawiono różnice frekwencji substytucji pojedynczych nukleotydów (SNP) w genach DRB3 i DQB głównego kompleksu zgodności tkankowej (MHC) odpowiedzialnych za odpowiedź immunologiczną w dwóch populacjach bytujących w Puszczy Białowieskiej w Białorusi i Polsce. Unikatowe i rzadkie allele, które są wartościowe dla populacji, których obecność i wzrost frekwencji może wpłynąć na podwyższenie poziomu zmienności genetycznej a tym samym żywotności gatunku zostały w populacjach zidentyfikowane.